Cloning and Expression in *Escherichia coli* of *Vibrio parahaemolyticus* Thermostable Direct Hemolysin and Thermolabile Hemolysin Genes

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Two hemolysin genes of *Vibrio parahaemolyticus* WP1, a thermostable direct (TSD) hemolysin gene and a thermolabile hemolysin gene, were cloned into the pBR322 vector in *Escherichia coli* K-12 C600. A large amount of the TSD hemolysin produced in *E. coli* K-12 accumulated in the periplasmic space. The TSD hemolysin gene was localized on a 0.9-kilobase HindIII-BamHI fragment by identifying qualitatively the production of the TSD hemolysin by a reverse passive hemagglutination assay in the osmotic shock fluid. The thermolabile hemolysin gene was isolated on a 1.3-kilobase HindIII-PstI fragment by selection with the hemolysin on blood agar. Southern blot hybridization and colony hybridization experiments indicated that the TSD hemolysin gene was present in the chromosomal DNA of 15 Kanagawa phenomenon-positive strains but not in 14 negative strains, whereas the thermolabile hemolysin gene was detected in all strains. No homologous DNA sequences to TSD and thermolabile hemolysin genes were detected in the chromosomes of *Vibrio cholerae*, *Vibrio vulnificus*, non-O1 *V. cholerae*, and *Vibrio anguillarum*.

*Vibrio parahaemolyticus* is a major cause of food poisoning, i.e., human acute gastroenteritis. This organism produces several hemolysins (35). One of them, the thermostable direct (TSD) hemolysin, hemolyzes erythrocytes on Wagatsuma medium (34), and this phenomenon has been called the Kanagawa phenomenon (Kp). Because the Kp is closely related to the enteropathogenicity of *V. parahaemolyticus*, it has been used for discriminating between pathogenic and nonpathogenic strains. However, there are some inconsistencies. The first inconsistency is the lack of correlation between the Kanagawa reaction of organisms isolated from patients with gastroenteritis and that of the incubated food source. Kp+ strains can be isolated from the feces of patients with gastroenteritis, whereas only Kp− strains have been isolated from vehicles of food poisoning (11, 19). The second inconsistency is that Kp+ organisms are not always reactive in the ligated rabbit ileal loop (24, 32). The third inconsistency is that Kp− strains are sometimes isolated from the stools of patients with gastroenteritis (14) and occasionally are the only strains of *V. parahaemolyticus* isolated (30). These facts place some doubt on the role of TSD hemolysin in enteropathogenicity.

For the purpose of a genetic study of pathogenicity, we first investigated the location of the TSD hemolysin gene. Because in *Escherichia coli* both hemolysin (27) and enterotoxin (26) genes often have been found on plasmids, the possible correlation between TSD hemolysin production and plasmid DNA was analyzed by using 37 *V. parahaemolyticus* strains (29). However, in agreement with the results of Guerry and Colwell (6) and Twedt et al. (33), no correlation was found between TSD hemolysin production and the presence of a plasmid, indicating that the TSD hemolysin gene is a chromosomal determinant.

As a continuation of this work, we cloned the hemolysin genes of *V. parahaemolyticus* into *E. coli*. We report here the results of cloning of two different hemolysin genes. A chromosomal DNA fragment (ca. 15 kilobases [kb] in length) which confers TSD hemolysin production has also been isolated recently by Kaper et al. (10).

MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** *V. parahaemolyticus* WP1, a Kp− isolate from a patient with gastroenteritis, was used as the source of chromosomal DNA for the cloning. Twenty-nine Kp+ or Kp− strains of *V. parahaemolyticus* from various sources were tested for the presence of TSD and thermolabile hemolysin genes. Six strains of *Vibrio cholerae*, five strains of *Vibrio vulnificus*, two strains of non-O1 *V. cholerae*, and one strain of *Vibrio anguillarum* were also used. *E. coli* K-12 C600 (lac, thr, leu, thi, tona, hspR, hspM) was used as a recipient for the transformation of recombinant plasmid DNA. The plasmids used were pBR322, pBR325, and pUC9. Nutrient agar (Nissui Seiyaku, Tokyo, Japan) and L-broth were used for growing all strains. Blood agar was prepared by adding 5 ml of packed washed human erythrocytes to 100 ml of 0.5% mannitol containing nutrient agar (mannitol-blood agar). Transformants were selected and maintained on nutrient agar medium supplemented with ampicillin (50 μg/ml), tetracycline (25 μg/ml), or chloramphenicol (20 μg/ml).

**Cloning procedures.** Whole cell DNA of *V. parahaemolyticus* WP1 was isolated from a 400-ml culture, purified as described by Saito and Miura (23), and digested completely with BamHI or PstI enzyme. The digested DNA was mixed with pBR322, which had been digested with the same enzyme and further dephosphorylated with the bacterial alkaline phosphatase (Millipore Corp, Freehold, N.J.) (17). This DNA mixture was ligated with T4 DNA ligase and used to transform *E. coli* K-12 C600. Transformants were selected on mannitol-blood agar supplemented with ampicillin in the case of digestion with BamHI or tetracycline in the case of digestion with PstI.

The procedures used for purification of large quantities of

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plasmid DNA (9, 15) and for agarose gel electrophoresis have been described previously (29). For the rapid screening of recombinant plasmids, DNA was isolated as described by Holmes and Quigley (7). Transformation of E. coli K-12 C600 with DNA was performed by the procedure of Ledeborg and Cohen (16). Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan), and used by the instructions of the suppliers.

Osmotic shock method. To release TSD hemolysin from E. coli, osmotic shock was performed as described by Neu and Heppel (20). Culture supernatant (supl) was obtained by centrifugation of a 20-ml overnight culture. Cell pellets were suspended in 1.6 ml of 20 mM sucrose–200 mM Tris–5 mM EDTA buffer, gently shaken for 10 min at room temperature, and then centrifuged. The supernatant (supl) was separated. Cell pellets were suspended in 1.6 ml of cold water, gently shaken for 10 min at 0°C, and then centrifuged. Supernatant (suplII) was isolated as an osmotic shock fluid.

Hemolysin assay. For quantitative and qualitative assay of the production of the TSD hemolysin, titration of hemolysin in the culture supernatant or osmotic shock fluid was carried out by reverse passive hemagglutination (RPHA) in microtiter wells with a KAP-KIT (Denka seiken, Niigata, Japan) (21).

Heat stability of the cloned hemolysin and the activity of the hemolysins against horse erythrocytes. The suplII fractions obtained from the osmotic shock treatment (see above) were treated at 60 or 100°C for 10 min and immediately chilled in an ice bath. Ten microliters of each sample was placed in a mannotol-blood agar well (3 mm in diameter), incubated at 37°C for 24 h, and observed for hemolysis. Hemolytic activity against horse erythrocytes was tested by the same method, except that non heated suplII fractions and 5% horse erythrocytes were used instead of human erythrocytes.

Southern blotting and colony hybridization analysis. The recombinant plasmids pUMTS61, pUMTS51, pUMT1, and pHLL421 were radiolabeled with [α-32P]dATP (Amersham Japan, Tokyo, Japan) in vitro by a previously described nick translation method (22) and used as probes. The method of transfer of DNA fragments from agarose gels to nitrocellulose filters (BA85; Schleicher & Schuell, Inc., GMBH) was as described by Southern (28). DNA-DNA hybridization was carried out by the procedure described by Denhardt (3). For colony hybridization, colonies which had been incubated on nutrient agar for 18 h were transferred to nylon membrane filters (BiodyneA; Nihon Paul Ltd., Tokyo, Japan), and DNA-DNA hybridization was carried out as described above.

RESULTS

Cloning in E. coli of V. parahaemolyticus hemolysin genes. From the mixture of BamHI digests of chromosomal DNA of V. parahaemolyticus WP1 and pBR322, one clone, which showed a clear hemolytic zone after 24 h at 37°C, was obtained from about 3000 clones and was designated HL1.

![FIG. 1. Physical map of pUMT1 and its derivatives. Symbols: ——, sequence of V. parahaemolyticus DNA subcloned into vector plasmid to form the corresponding plasmids; ☐, sequence of vector pBR322; ☐☐, sequence of vector pUC9. KAP-KIT RPHA. RPHA titer with anti-TSD hemolysin antibody-sensitized rabbit erythrocytes; B, BamHI; E, EcoRI; P, PstI; RV, EcoRV; H, HindIII; S, SalI; Sm, SmaI.](http://jb.asm.org/Downloaded from http://jb.asm.org)
parahaemolyticus was obtained from about 3000 clones. UMT1 did not show any detectable hemolysis after 24 h at 37°C, but showed a very turbid hemolytic zone after standing for 48 h at 4°C. The recombinant plasmids harbored by HL1 and UMT1 were designated pH1L and pUMT1, respectively.

Characterization of the hemolysins produced by HL1 and UMT1. The production of TSD hemolysin in the UMT1 clone was detected by the RPHA method (Table 1). Although the titer of the culture supernatant of the UMT1 clone (supl of C600[pUMT1]) was low (four times); that of the osmotic shock fluid (supIII of C600[pUMT1]) was very high. This indicates that the TSD hemolysin gene of V. parahaemolyticus was expressed in E. coli cells, and most (ca. 80%) of the TSD hemolysin synthesized in E. coli cells accumulated in the periplasm. In contrast, V. parahaemolyticus cells secreted almost all (>99%) of the TSD hemolysin into the culture medium, and less than 1% of the hemolysin was retained in the periplasm. The quantity of the TSD hemolysin in the cytoplasm of E. coli could not be measured because of the nonspecific hemagglutination of erythrocytes by the cell extract of E. coli.

The RPHA titers of supI and supIII of the HL1 clone were less than 2, suggesting that the clone might produce thermolabile hemolysin. To substantiate this possibility, heat stability and hemolytic activity of the two hemolysins against human and horse erythrocytes were determined. The hemolytic activity of osmotic shock fluid of UMT1 was resistant to heat treatment (100°C, 10 min) and was clearly detectable with human erythrocytes but not with horse erythrocytes (data not shown), which is characteristic of TSD hemolysin. However, the hemolytic activity of hemolysin produced by HL1 was destroyed by heat treatment (60 or 100°C, 10 min), and the activity was clearly detectable with human erythrocytes. This thermolabile hemolysin also showed activity against horse erythrocytes, although the reaction with horse erythrocytes was slow and the hemolytic zones were turbid compared with the reaction with human erythrocytes.

Subcloning of the TSD hemolysin gene on pUMT1. Subclones and deletion derivatives of pUMT1 were isolated to locate the TSD hemolysin gene on the 12.5-kb fragment (Fig. 1). The TSD hemolysin productivity by the derivatives was assayed by RPHA titration of the osmotic shock fluid. A subclone containing a 5.7-kb SalI fragment (pUMT2bl) produced TSD hemolysin. Both of the two deletion derivatives of pUMT2bl (pUMT128 which was generated by deletion of a 2-kb SalI-EcoRI fragment and pUMT3130 lacking a 3.7-kb SalI-BamHI fragment) also produced TSD hemolysin.

To narrow down the TSD hemolysin determinant, a restriction map of pUMT3130 was constructed (Fig. 2). The cloned fragment carried one site for HincII and two sites for HindIII. Subclones and deletion derivatives of pUMT3130 were isolated as shown in the figure, and, finally, the TSD hemolysin gene was mapped on the 0.9-kb HindIII-BamHI fragment. The HincII cleavage site was mapped in the TSD hemolysin gene, because the transformants that possessed pUMT541, pUMT551, or pUMT561 did not produce the hemolysin.

![FIG. 2. Additional subcloning of the TSD hemolysin gene in pUMT3130. Symbols are the same as described in the legend to Fig. 1.](http://jb.asm.org/)

![FIG. 3. Physical map of pH1L and its derivatives. Symbols: —, sequence of the subcloned DNA of V. parahaemolyticus into vector plasmids to form the corresponding plasmid; ■, thermolabile hemolysin gene; Hly, hemolysis on mannitol-blood agar; III, HindIII.](http://jb.asm.org/)
TABLE 2. Colony hybridization* of V. parahaemolyticus and other vibrios with probes pUMT31, pUMT1, and pHL421

<table>
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<th>Source</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>pUMT31</td>
</tr>
<tr>
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<td>+</td>
<td>13</td>
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<td>+</td>
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<td>5</td>
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<td>V. anguillarum</td>
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</table>

* Method of colony hybridization is described in the text.

† Patients with gastroenteritis.

‡ One of five strains was a Kp+ mutant, which was isolated from a Kp- strain after overnight culture. Three strains had been Kp+ at primary isolation, but all populations of them changed to Kp- during storage. The other strain was isolated from the stool of a patient with gastroenteritis.

§ Two of five strains hybridized more weakly than did the positive control.

Subcloning of thermolabile hemolysin gene on pHL1. pHL1 DNA contained a 12.5-kb BamHI fragment inserted into pBR322. The restriction endonuclease digestion map is shown in Fig. 3. Because the hemolysis around E. coli cells carrying pHL1 was clear after incubation for 24 h, the hemolysis on the blood agar was used as a marker for selection of the derivatives. The cloned 12.5-kb fragment of pHL1 DNA had five PstI and two EcoRI sites, and a 2.35-kb BamHI-PstI fragment was shown to have the hemolysin gene. This fragment had two HindIII sites, and a deletion derivative, plasmid pHL421, lacking only the central HindIII fragment was hemolysin positive, whereas pHL422, with a left-hand BamHI-HindIII fragment, was negative. The results indicated that the thermolysin hemolysin gene is located on the 1.3-kb HindIII-PstI fragment.

Hybridization of the TSD or thermolabile hemolysin sequence with chromosomal DNA of Kp+ or Kp- V. parahaemolyticus and strains of other Vibrio species. To determine whether Kp+ or Kp- V. parahaemolyticus strains derived from different sources possessed TSD or thermolabile hemolysin genes, the cloned TSD or thermolabile hemolysin sequences were labeled by nick translation and used for hybridization with chromosomal DNA isolated from WP1 (the parent strain of the cloning), WP28 (a Kp- strain isolated from the causative food source), K44 (a Kp+ strain isolated from a patient with gastroenteritis), and K6 (a Kp- strain from a patient with gastroenteritis). Figure 4A shows the results of the Southern blot hybridization in which pUMT561 was used as a probe (pUMT561 possessed part of the TSD hemolysin gene). The two Kp- strain WP1 and K44 (lanes 1, 2, 5, and 6; Fig. 4A) had an homologous sequence, whereas the Kp+ strains WP28 (lanes 3 and 4, Fig. 4A) and K6 (lanes 7 and 8, Fig. 4A) did not, although K6 was isolated from a patient with gastroenteritis.

When pHL421, which carries the thermolysin hemolysin gene, was used as a probe, it was revealed that all four strains possessed sequences homologous with the thermolysin gene (Fig. 4B). There was no homology between these two hemolysin genes (data not shown).

To provide more information on the distribution of the hemolysin genes, 28 V. parahaemolyticus strains obtained from various sources, 6 strains of V. cholerae, 5 strains of V. vulnificus, 2 strains of non-O1 V. cholerae, and 1 strain of V. anguillarum were subjected to colony hybridization, with pUMT531, pUMT1, and pHL421 used as DNA probes. Because pUMT1 contains relatively large V. parahaemolyticus DNA, the homology with neighboring DNA sequences of the TSD hemolysin locus also can be detected (Table 2). All V. parahaemolyticus strains tested possessed DNA sequences homologous to pHL421 (which includes the thermolysin hemolysin gene). All Kp+ strains (13 from clinical isolates and 1 from a causative food source) gave positive hybridization signals with both pUMT531 and pUMT1. In addition to the four Kp- strains obtained from environmental sources (one from seawater and three from causative seafood), five Kp- strains from patients with gastroenteritis...
V. parahaemolyticus has been reported to produce several hemolysins (35), one of which is a TSD hemolysin closely related to human enteropathogenicity (11, 19). In particular, strain WP1, a parent strain used for cloning in this study, has been reported to produce two hemolysins (4, 18).

In this study, the TSD hemolysin and thermodible hemolysin genes were cloned into a vector plasmid in E. coli K-12. Because E. coli K-12 does not grow on Wagatsuma medium, because the medium contains 3% NaCl, the transformants were selected on mannitol-blood agar. On this medium, not only TSD hemolysin but also other thermodible hemolysins can produce hemolytic zones. Therefore, RPHA titration with rabbit erythrocytes sensitized with anti-TSD hemolysin antibody was necessary to distinguish the TSD hemolysin from other hemolysins.

In contrast to V. parahaemolyticus, the amount of cell-free TSD hemolysin released from E. coli harboring the recombinant plasmid was small. More than 99% of TSD hemolysin synthesized in V. parahaemolyticus was released into supernatant fluid, whereas only about 15% of it was released into the culture supernatant in E. coli. Similar to the production of the enterotoxin of V. cholerae (5) in E. coli, most of the TSD hemolysin synthesized in E. coli appeared to accumulate in the periplasmic space. The quantity of the TSD hemolysin in the cytoplasm of E. coli could not be measured because of the nonspecific hemagglutination of erythrocytes by the cell extract of E. coli. Association of the gene product in the E. coli cytoplasm has been found in phospholipase C of Pseudomonas aeruginosa (2) and α-hemolysin of Staphylococcus aureus (12). In P. aeruginosa, however, the amount of the enzyme in the periplasmic space was very small.

The TSD hemolysin determinant was localized in a small region of DNA of ca. 900 base pairs. This region also contains the regulatory sequences and is sufficient to cover the structural gene of TSD hemolysin, because the TSD hemolysin gene consists of 163 amino acids (31), which corresponds to 489 base pairs. The molecular weight of thermodible hemolysin is not known; however, the gene for the hemolysin was also localized to a small region of approximately 1.3 kb. More detailed analysis will be required to determine the precise structure of the thermodible hemolysin determinant.

Recently, Kaper et al. (10) have succeeded in cloning the TSD hemolysin determinant into an approximately 15-kb fragment of V. parahaemolyticus. By colony hybridization, they showed that none of the Kp + environmental isolates possessed DNA sequences homologous to the fragment. In contrast, our data indicate that five Kp + strains of nine environmental isolates contained sequences homologous to the pUM1 probe (which carried a 12.5-kb fragment, including the TSD hemolysin gene of strain WP1), although they did not contain the TSD hemolysin locus. The results suggest that a variable genetic element is involved in this region of the V. parahaemolyticus chromosome.

DISCUSSION

The nature of thermodible hemolysin cloned into pHL1 is obscure, because our thermodible hemolysin is active against not only human erythrocytes but also horse erythrocytes, whereas the thermodible direct hemolysin of strain WP1 reported by Fujino et al. (4) is inactive to horse erythrocytes (18). The reason for this is not clear. Presumably, the cloned hemolysin determinant used in this study is different from that reported by Fujino et al. (4). In contrast to TSD hemolysin, the thermodible hemolysin gene was detected in all of the strains tested, regardless of their history. This result was in accordance with the results of Sakurai et al. (25). The role of this hemolysin in the enteropathogenicity of V. parahaemolyticus is not known.

Results of our preliminary hybridization experiments indicate that there are no homologous sequences between cloned fragments and chromosomal DNAs of other vibrios. However, it will be of interest to use these plasmids for phylogenetic studies.

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LITERATURE CITED


